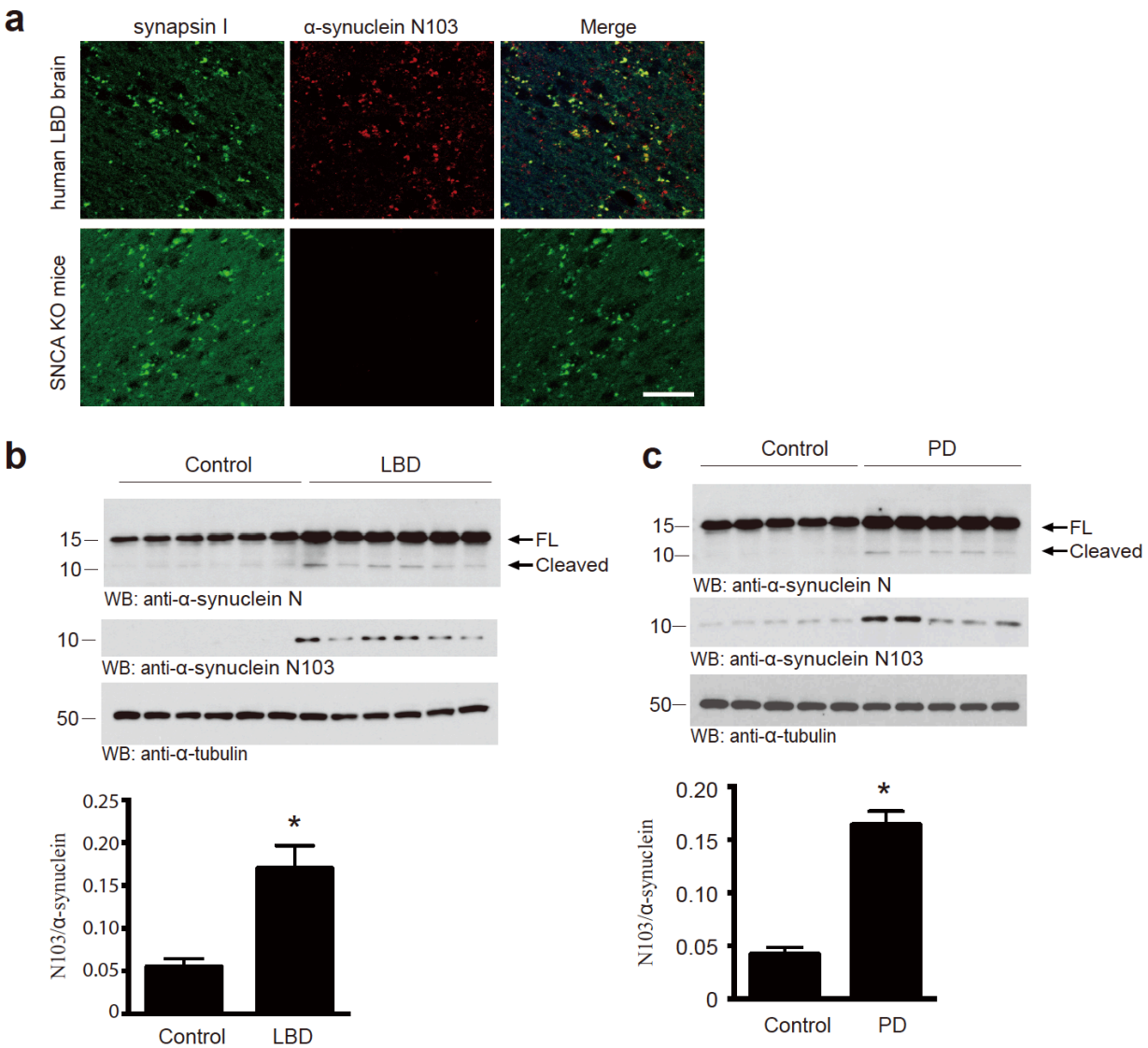
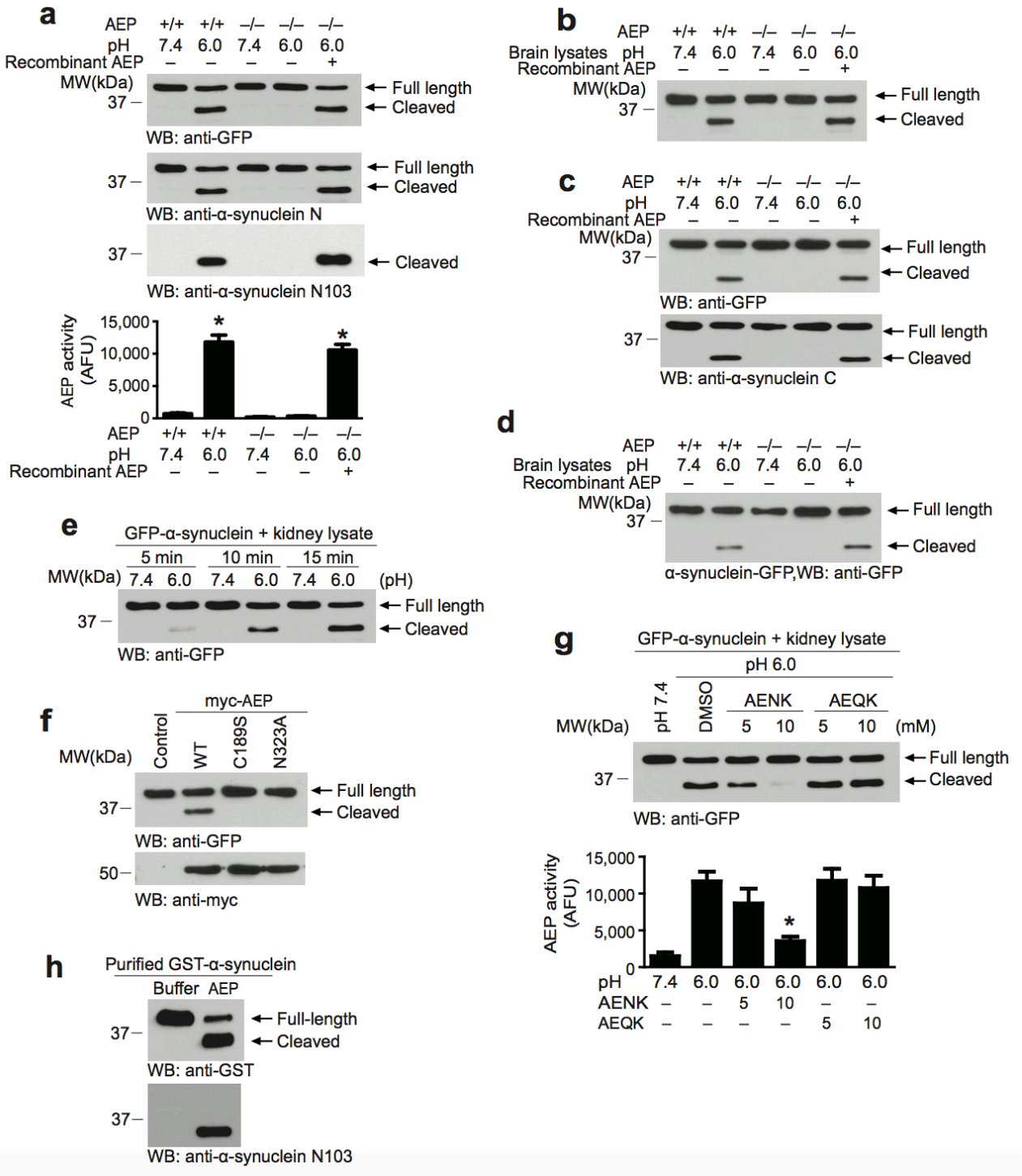
Asparagine Endopeptidase Cleaves α-synuclein and Mediates its Pathologic Activities in Parkinson's Disease

By

Zhentao Zhang, Seong Su Kang, Xia Liu, Zhaohui Zhang, Li He, P. Michael Iuvone, Duc M. Duong, Nicholas T. Seyfried, Matthew J Benskey, Fredric P. Manfredsson, Lingjing Jin, Yi E. Sun, Jian-zhi Wang and Keqiang Ye

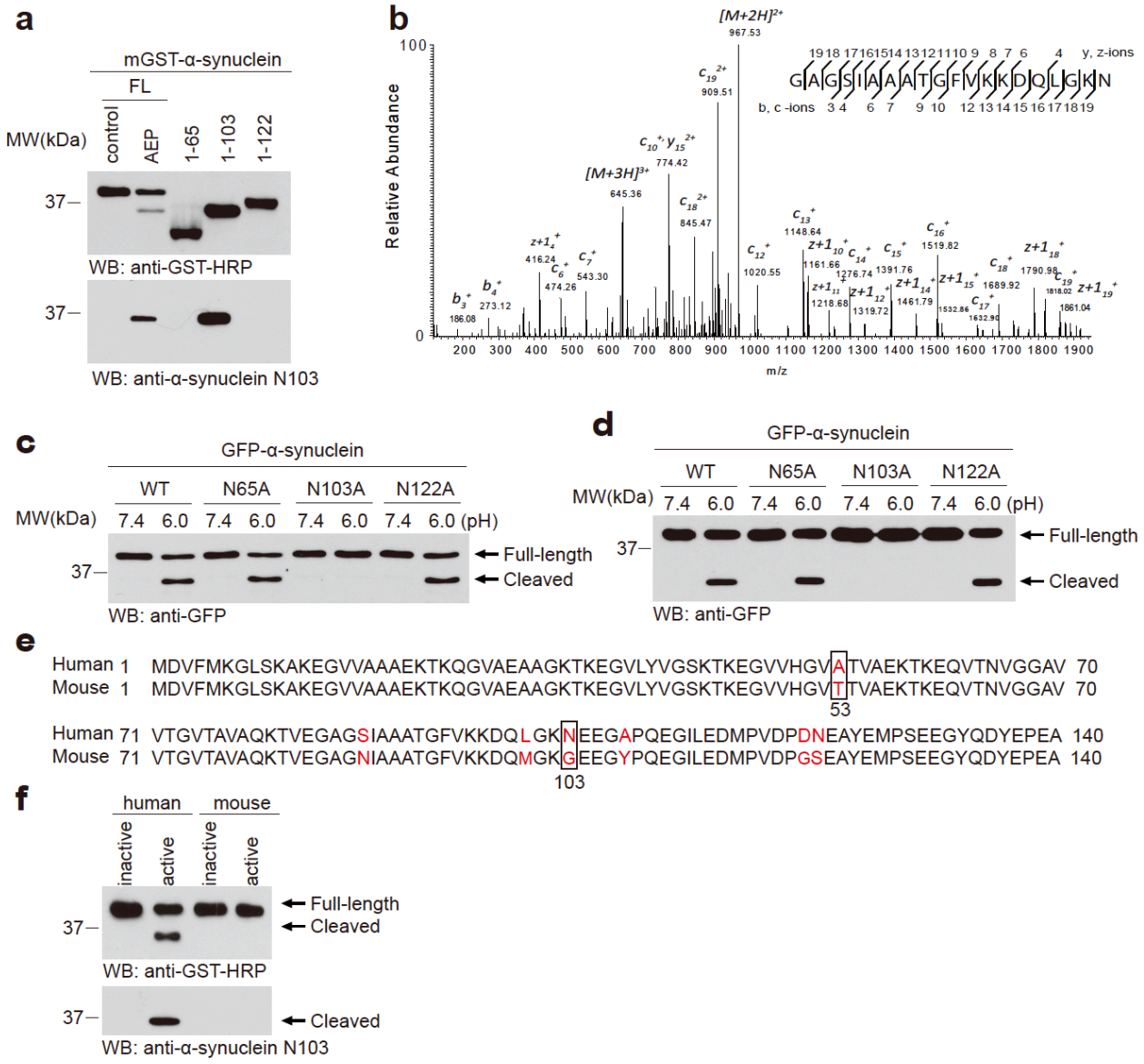


Supplementary Figure 1. α-synuclein is truncated in PD and LBD brain. (**a**) Localization of α-synuclein N103 fragments in synaptic structures. LBD brain sections were immunostained with anti-α-synuclein N103 (red) and presynaptic marker synapsin I (green). Scale bar, 20 μm. Images are representative of 9 sections from three subjects. SNCA knockout mice brain was used as negative control. (**b-c**) Western blot showing the presence of α-synuclein N103 fragment in brain lysates from human LBD cortex and PD SN tissues (mean ± SEM; $n = 6$, $^*P < 0.05$ compared with control, student's *t*-test). The shown blots are the representative figures of three independent experiments.



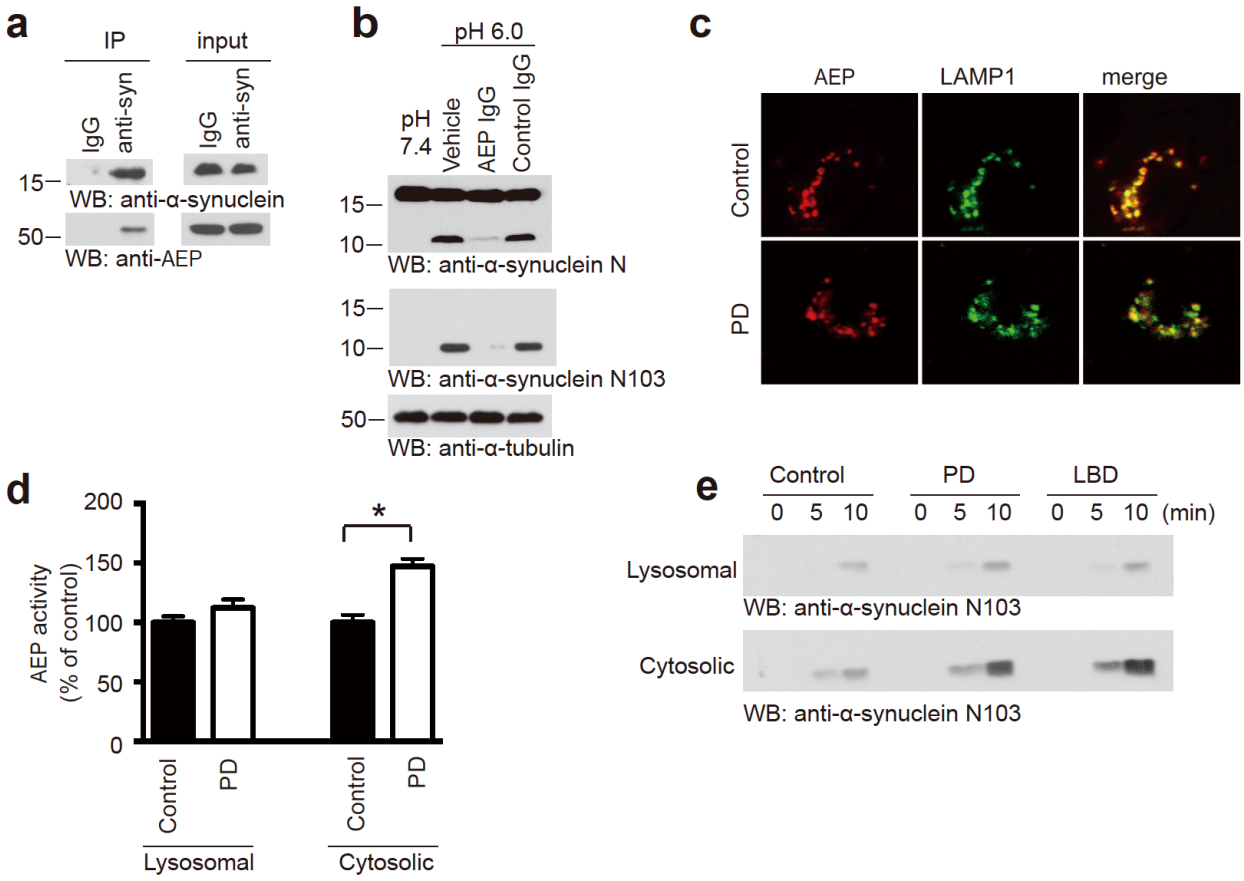
Supplementary Figure 2. α-synuclein is a substrate of AEP. (**a**) α-synuclein cleavage assay in kidney lysates. N-terminal GFP-tagged α-synuclein was incubated with kidney lysates from WT or AEP knockout mice at pH 7.4 or pH 6.0 at 37°C for 15 min. Western blot shows that α-synuclein was cleaved at pH 6.0 (upper panel) when AEP was activated (lower panel) (mean ± SEM; n =3, *P < 0.05 compared with pH 7.4 group, one-way ANOVA). (**b**) α-synuclein cleavage assay in

brain lysates. N-terminal GFP-tagged α-synuclein was incubated with brain lysates from WT or AEP knockout mice at pH 7.4 or pH 6.0 at 37°C for 15 min. (**c-d**) Processing of C-terminal GFP-tagged α-synuclein in mouse kidney lysates (**c**) and brain lysates (**d**). (**e**) Time-dependent cleavage of α-synuclein by AEP. (**f**) α-synuclein cleavage by wild-type and mutant AEP. (**g**) The proteolysis of α-synuclein is blocked by AENK peptide, but not AEQK (upper panel). The effect of AENK on AEP was confirmed by enzymatic activity assay (bottom panel) (mean ± SEM; n =3, *P < 0.05 compared with wild-type group, one-way ANOVA). (**h**) Purified active recombinant AEP potently cleaves purified GST-α-synuclein recombinant protein. The AEP-generated α-synuclein fragment was recognized by anti-α-synuclein N103 antibody. The shown blots are the representative figures of three independent experiments.



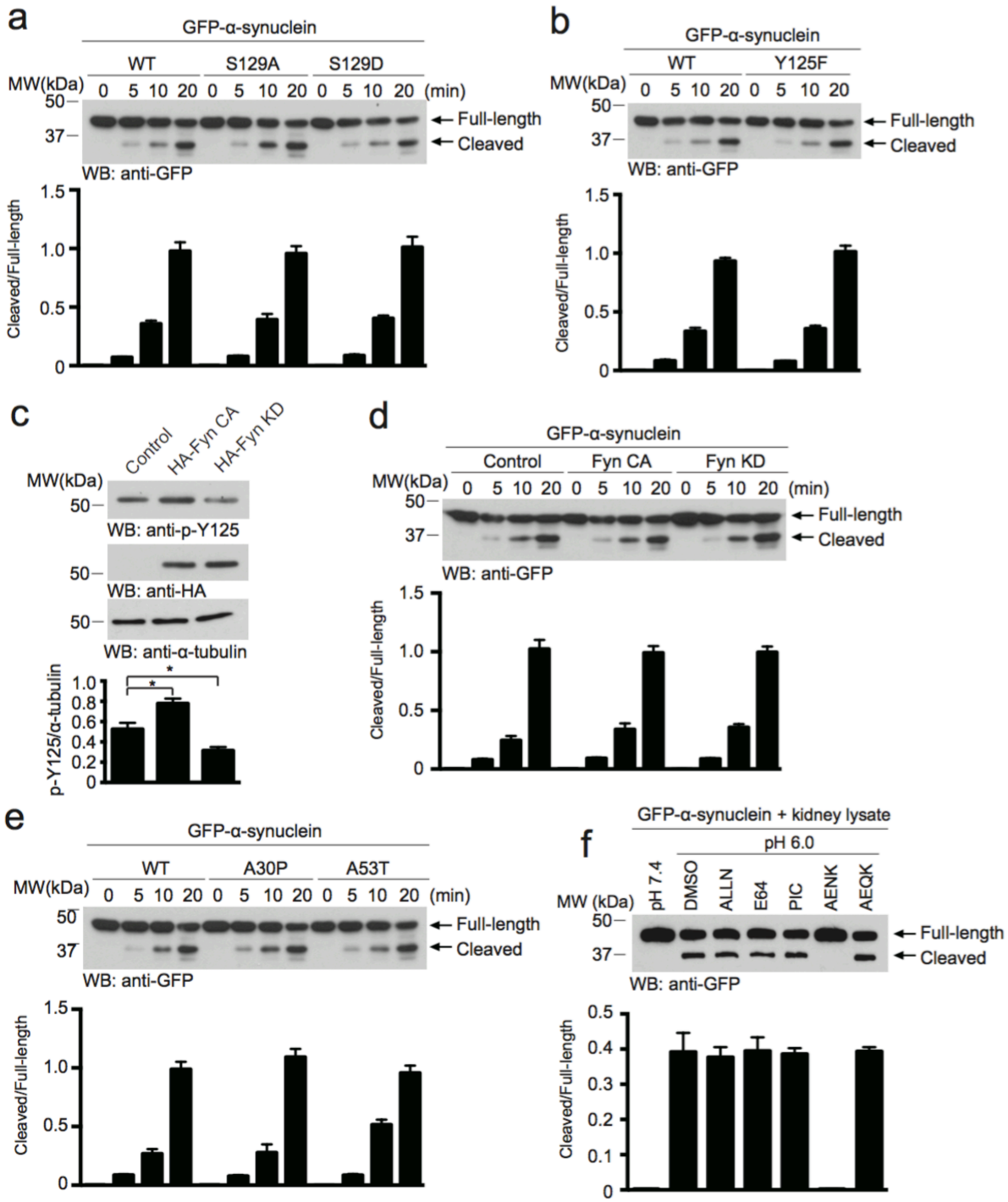
Supplementary Figure 3. α-synuclein is cleaved at N103 by AEP. (**a**) Recombinant mammalian glutathione transferase (mGST)-tagged α-synuclein was incubated with purified active AEP, and analyzed by immunoblotting. The AEP-derived α-synuclein fragment shows the same molecular weight as a.a. 1-103 fragment. The AEP-derived α-synuclein fragment was recognized by the anti-α-synuclein N103 antibody. (**b**) MS/MS spectrum showing that AEP cleaves α-synuclein after N103 *in vitro*. The purified mGST-α-synuclein was incubated with active AEP for 30 min. The fragment was subject to MS/MS spectrum assay. (**c-d**) Cleavage of mutant N-terminal GFP-tagged α-synuclein or C-terminal GFP-tagged α-synuclein by AEP. α-synuclein cleavage was analyzed by

Western blot after recombinant α-synuclein wide-type, N65A, N103A, or N122A mutants were incubated with active mouse kidney lysates for 15 min. (**e**) Comparison of protein sequences of human and mouse α-synuclein. (**f**) Cleavage of human and mouse α-synuclein by recombinant AEP. The shown blots are the representative figures of three independent experiments.



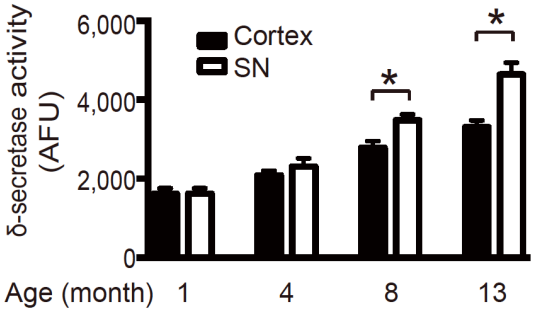
Supplementary Figure 4. AEP interacts with α-synuclein. (**a**) Co-immunoprecipitation of α-synuclein and AEP in PD brain samples. α-synuclein was immunoprecipitated with anti-α-synuclein N-terminal antibody, and analyzed by immunoblotting with anti-AEP antibody. (**b**) Anti-AEP antibody abolishes the cleavage of α-synuclein by AEP. Anti-AEP antibody or control IgG was added into human brain lysates and incubated at pH 6.0 for 15 min. The proteolytic processing of α-synuclein was analyzed using Western blot. The shown blots are the representative figures of three independent experiments. Data represent mean ± SEM of three experiments. n = 3, *P < 0.05, one-way ANOVA. (**c**) Immunostaining with lysosomal marker LAMP1 and δ-secretase showing strict lysosomal localization of δ-secretase in the control brain slides and diffuse staining of δ-secretase in PD brain slides. (**d**) δ-secretase activity in lysosomal and cytoplasmic fractions of control and PD brain samples. (mean ± SEM; n = 3, *P < 0.05, one-way ANOVA) (**e**) Cleavage of recombinant α-synuclein by lysosomal and cytoplasmic

fraction. Recombinant α-synuclein was incubated in lysosomal and cytoplasmic fraction of human brain tissue at pH 6.0 for 0, 5 and 10 min. The production of α-synuclein N103 fragment was analyzed using Western blot.

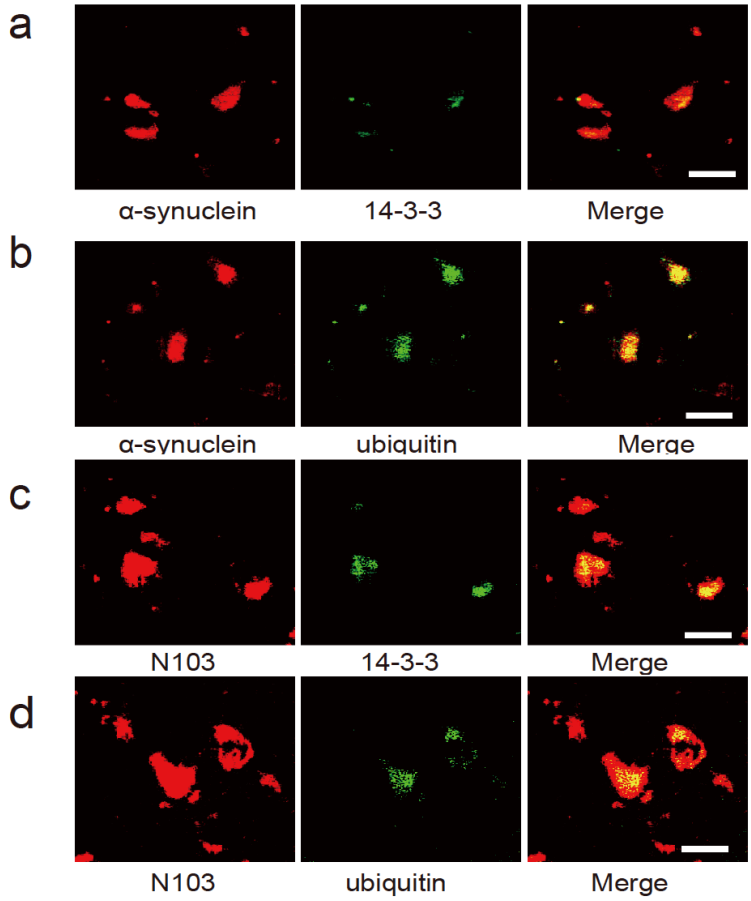


Supplementary Figure 5. The cleavage of α-synuclein by AEP is independent of its phosphorylation and mutations. (**a**) Cleavage rate of α-synuclein S129A and S129D mutations by AEP. HEK293 cells were transfected with GFP-α-synuclein wild-type, S129A, and S129D mutations of α-synuclein, and incubated with active kidney lysates for 0, 5, 10, or 20 min at pH 6.0, and analyzed by immunoblotting. (**b**) Cleavage rate of α-synuclein Y125F mutation by AEP. (**c**)

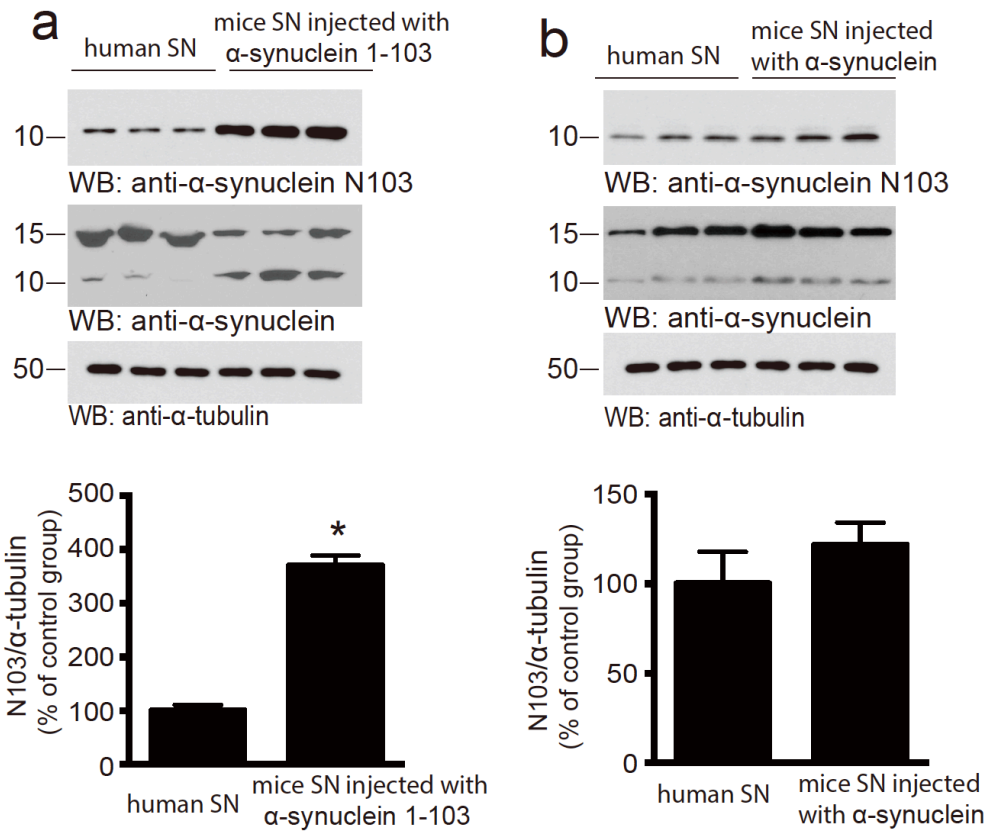
Overexpression of constitutively active Fyn induced the phosphorylation of α-synuclein at Y125. (**d**) Cleavage assay indicates that constitutively active (CA) Fyn or kinase dead (KD) Fyn does not affect the cleavage rate of α-synuclein. (**e**) A30P and A53T mutant α-synuclein were cleaved by AEP at a similar rate as wild-type (WT) α-synuclein. (**f**) Cleavage of α-synuclein by AEP was not blocked by calpain, cathepsin or protease inhibitor cocktail. HEK293 cells were transfected with GFP-α-synuclein. The cell lysates were incubated with active kidney lysates at 37°C for 10 min in the presence of calpain inhibitor ALLN, cathepsin inhibitor E64, protease inhibitor cocktail, or AEP inhibitor AENK. α-synuclein cleavage was analyzed by Western blot. Only AEP inhibitory peptide AENK but not any other small molecular inhibitors antagonized α-synuclein cleavage by AEP. The shown blots are the representative figures of three independent experiments. The shown blots are the representative figures of three independent experiments. Data represent mean ± SEM of three experiments. $*P < 0.05$, one-way ANOVA.



Supplementary Figure 6. AEP activity assay in wild-type mice. AEP activity is escalated in the cortex and SN tissues of wild-type mice in an age-dependent style (mean ± SEM; $n = 6$, $^*P < 0.05$, one-way ANOVA). AFU, arbitrary fluorescence units.



Supplementary Figure 7. Expression of α-synuclein full-length or 1-103 fragments induces intraneuronal inclusions containing ubiquitin and 14-3-3. Brain slides injected with AAVs encoding α-synuclein full-length (a, b) or 1-103 (c, d) were immunostained with α-synuclein antibody (a, b), anti-α-synuclein N103 antibody (c, d), 14-3-3 antibody (a, c) and ubiquitin antibody (b, d).



Supplementary Figure 8. Expression level of N103 fragments. Western blot shows the expression level of α-synuclein 1-103 fragments in SN tissues from PD patients, mice SN tissue injected with human α-synuclein virus, and mice SN tissue injected with α-synuclein 1-103 fragment virus (mean ± SEM; $n = 3$, $^*P < 0.05$, student's *t*-test).